Protein kinase CK2 phosphorylates Hsp105α at Ser^{509} and modulates its function

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The 105 kDa heat-shock protein (Hsp) Hsp105α is a mammalian stress protein that belongs to the HSP105/HSP110 family. We have shown previously that Hsp105α exists as non-phosphorylated and phosphorylated forms *in vivo*, and is phosphorylated by protein kinase CK2 (CK2) *in vitro*. In this study, to elucidate the role of phosphorylation of Hsp105α, we first analysed the site of phosphorylation of Hsp105α by CK2. Peptide mapping analysis of Hsp105α phosphorylated by CK2 and *in vitro* phosphorylation experiments using various deletion and substitution mutants of Hsp105α revealed that Hsp105α is phosphorylated at Ser^{509} in the β-sheet domain. Furthermore, Ser^{509} in Hsp105α was also phosphorylated in mammalian COS-7 cells, although other sites were phosphorylated as well. Next, we examined the effects of phosphorylation of Hsp105α on its functions using CK2-phosphorylated Hsp105α. Interestingly, Hsp105α suppressed 70 kDa heat-shock cognate protein (Hsc70)-mediated protein folding, whereas the phosphorylation of Hsp105α at Ser^{509} abolished the inhibitory activity of Hsp105α *in vitro*. In accordance with these findings, wild-type Hsp105α, which was thought to be phosphorylated *in vivo*, had no effect on Hsp70-mediated refolding of heat-denatured luciferase, whereas a non-phosphorylatable mutant of Hsp105α suppressed the Hsp70-mediated refolding of heat-denatured luciferase in mammalian cells. Thus it was suggested that CK2 phosphorylates Hsp105α at Ser^{509} and modulates the function of Hsp105α. The regulation of Hsp105α function by phosphorylation may play an important role in a variety of cellular events.

Key words: chaperone activity, heat-shock protein 70 (Hsp70).

INTRODUCTION

Heat-shock proteins (Hsps), also called stress proteins, are expressed under both stressed and physiological conditions. Most Hsps serve not only to protect cells from the cytotoxic effects of various stressors, but also play important roles in normal cellular functions as molecular chaperones that regulate the conformation and activity of a variety of cellular proteins [1–3]. Mammalian Hsps are classified into several families: HSP27, HSP60, HSP70, HSP90 and HSP105/HSP110. The HSP70 family is the major and best-characterized group of Hsps, and most eukaryotes have at least several different species of Hsp70 in a variety of cellular compartments. HSP70-family proteins play a key role in folding, translocation and degradation of cellular proteins. HSP70-family proteins are commonly composed of three functional domains [4]. The highly conserved N-terminal ATPase domain binds ADP/ATP and can hydrolyse ATP, the central β-sheet domain directly binds peptide substrates and the C-terminal α-helix domain regulates its substrate binding [4–6]. The chaperone activity of Hsp70 relies on the cycling of binding and release of substrates, and several proteins such as Hdj-1 [7], Hip [8], CHIP [C-terminus of 70 kDa heat-shock cognate protein (Hsc70)-interacting protein] [9] and BAG-1 (Bcl-2-interacting protein) [10] have been characterized as co-chaperones of Hsp70.

In contrast, although cloning of HSP105/HSP110-family proteins has been reported in humans, mice, hamsters, plants, fungi and yeasts [11–17], their functions and crucial roles in cells are still not well understood. We have characterized two high-molecular-mass stress proteins, Hsp105α and Hsp105β, which belong to the HSP105/HSP110 family [18,19]. Hsp105α is expressed constitutively but is also induced by various stresses in most tissues of mammals and mammalian cell lines. On the other hand, Hsp105β is specifically induced by heat shock at 42 °C in mammalian cells, and is an alternatively spliced form of Hsp105α [11,12,20]. Hsp105α and Hsp105β are composed of N-terminal ATP binding, central β-sheet, loop and C-terminal α-helix domains similarly to the HSP70-family proteins. Both proteins exist as complexes associated with Hsp70 and Hsc70 (a constitutive member of the HSP70 family) in mammalian cells [21,22]. Furthermore, as Hsp105α and Hsp105β suppress the Hsc70 chaperone activity, these proteins were suggested to function as negative regulators of Hsc70 [23].

Both Hsp105α and Hsp105β consist of two isoforms, an acidic and a basic isoform, in mammalian cells [24]. These were found to be phosphorylated and non-phosphorylated isoforms, and protein kinase CK2 (CK2) phosphorylated these proteins *in vitro* [25]. Hsp105α is expressed in most rat and mouse tissues, and the protein is expressed at especially high levels in the brain [26]. Furthermore, non-phosphorylated and phosphorylated forms of Hsp105α are expressed at approximately similar levels in most tissues, although the phosphorylated form of Hsp105α is expressed at much higher levels than the non-phosphorylated form in the brain [26]. Recently, Hsp105α was shown to prevent stress-induced apoptosis in neuronal cells and was suggested to play important roles in neuronal protection in the brain [27].

In this study, to elucidate the role of phosphorylation of Hsp105α, we determined the phosphorylation site and the effects of phosphorylation on the function of Hsp105α. Our results revealed that CK2 phosphorylates Ser^{509} in the β-sheet domain and modulates the function of Hsp105α.

Abbreviations used: Hsp, heat-shock protein; Hsc70, 70 kDa heat-shock cognate protein; CK2, protein kinase CK2; Hsp105S509A etc., Ser^{509} → Ala mutant of Hsp105 etc.; Lys-C, lysyl endopeptidase; CBB, Coomassie Brilliant Blue R-250.

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EXPERIMENTAL

Cell culture

Monkey kidney COS-7 cells expressing simian virus 40 large T antigen (supplied from Riken Gene Bank) were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum in a 5 % CO_2 atmosphere at 37 °C.

Plasmids

For construction of deletion mutants of Hsp105α expression plasmids, the expression plasmid for full-length mouse Hsp105α with a His tag (pTrcHis105-1) [23] was digested with *Hin*dIII, and the resultant 1.3 and 1.2 kb fragments were cloned into the complementary site of the pTrcHisA expression vector (Invitrogen), and designated Hsp105N (residues 1–443) and Hsp105C (444–858), respectively. For other Hsp105α deletion mutants, PCR was performed with pTrcHis105-1 as a template using specific primers (underlines indicate additional *Kpn*I sites): Hsp105N2 (residues 1–511), sense primer, 5′-GGGGTACC-CAGCCATGTCGGTGGTT-3′, and antisense primer, 5′-GG-GGTACCTGGTTTGGACATTCCATGT-3′; Hsp105N3 (1–606), sense primer, 5′-ACTGCCATACCAAGTTGG-3′, and anti-sense primer, as for Hsp105N2; Hsp105C1 (605–858), sense primer, 5′-GGGGTACCAGTTAGGGAGAGACCTT-3′, and anti-sense primer, 5′-AATCTTCTCTCATCCGCC-3′; Hsp105C2 (511–858), sense primer, 5′-GGGGTACCAGGGAGAGACCTT-CTT-3′, and antisense primer, as for Hsp105C1; Hsp105C3 (386–858), sense primer, 5′-GGGGTACCCGGCATTTAAAG-TTAGAGAG-3′, and antisense primer, as for Hsp105C1. The PCR products were purified, digested with *Kpn*I and then cloned into the complementary site of the pTrcHisA expression vector. Substitution mutants of Hsp105α in which Ser at position 496, 509 or 526 was substituted with alanine were made using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The primers used for site-directed mutagenesis were as follows: 5′-ACCATCTCCAC-GGCCGCCATGGTGGAGAAGGT-3′ (S496A sense), 5′-AC-CTTCTCCACCATGGCGGCCGTGGAGATGGT-3′ (S496A anti-sense), 5′-CGAGGAAGAGGATGGCGCCTCTCTCGAGG-CAGA-3′ (S509A sense), 5′-TCTGCCTCGAGAGAGGCGCC-ATCCTCTTCCTCG-3′ (S509A antisense), 5′-AGGCCAACAG-AAAGCGCTGATGTGGATAAAAATATCC-3′ (S526A sense) and 5′-GGATATTTTTATCCACATCAGCGCTTTCTGTTGGC-CT-3′ (S526A antisense). Sequences were confirmed by automated DNA sequencing. All fusion constructs were transformed into *Escherichia coli* BL21.

For construction of mammalian expression plasmids of wild-type Hsp105α and Hsp105S509A, pTrcHis105-1 and pTrcHis105S509A were digested with *Bam*HI and *Eco*RI, cloned into the complementary site of the pcDNA3 expression vector (Invitrogen) and designated pcDNA105WT and pcDNA105S509A, respectively. Mammalian expression plasmid for human Hsp70 (pCMV70) [28] was a gift from Dr. K. Ohtsuka (Chubu University, Kasugai, Japan). Plasmid pGL2-control vector (Promega) was used for expression of firefly luciferase in mammalian cells.

Protein purification

Recombinant His-tagged proteins (wild-type and mutant Hsp105α and Hdj-1) were purified by affinity chromatography on Ni^{2+} resin (Invitrogen) as described previously [23]. The proteins eluted from Ni^{2+} resin were further purified by Mono Q anion-exchange column chromatography (gel volume, 1 ml; Amersham Biosciences). Proteins were loaded on to the Mono Q column pre-equilibrated with buffer M (25 mM Tris/HCl, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM EDTA and 20 mM NaCl) and washed with 30 ml of buffer M. The bound proteins were eluted with 50 ml of a linear gradient of 20–350 mM NaCl in buffer M, and the purified proteins were concentrated, dialysed against buffer M and stored at −80 °C.

Hsc70 was purified from bovine brain by successive DEAE-Sepharose CL6B and ATP–agarose column chromatography, as described previously [29].

In vitro phosphorylation of Hsp105α by CK2

Wild-type or mutant Hsp105α (10 pmol each) was incubated with 10 units of CK2 (Sigma) and 50 μM [γ-^{32}P]ATP (5 Ci/mmol) in 20 μl of CK2 buffer (20 mM Hepes-KOH, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 200 mM NaCl, 20 mM β-glycerophosphate and 20 mM NaF) for 3 h at 37 °C. The reaction was terminated by addition of 4 × SDS-Laemmli sample buffer, and phosphorylated proteins were separated by SDS/PAGE and detected by autoradiography.

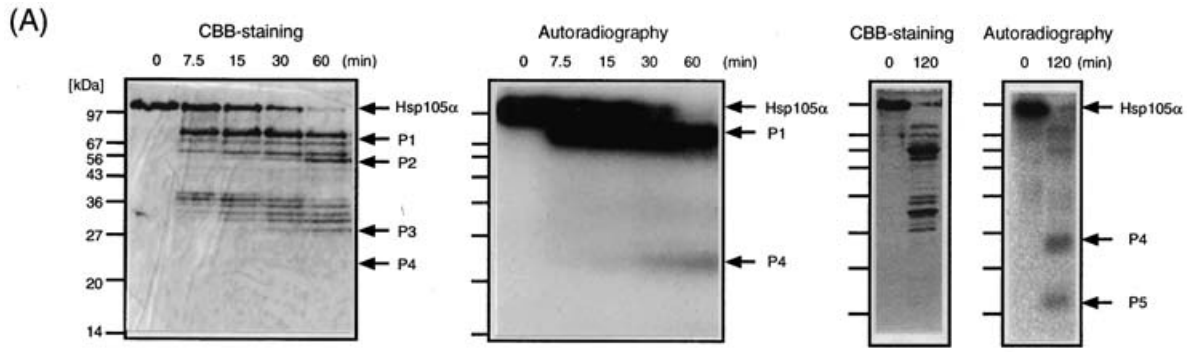
For preparation of CK2-phosphorylated Hsp105α, Hsp105α was incubated with CK2 in the presence of 200 μM ATP at 37 °C for 3 h. After incubation, the reaction mixture was subjected to gel-filtration chromatography on a Sephadex G-50 column to remove free excess ATP. To determine the stoichiometry of phosphorylation of Hsp105α, Hsp105α was phosphorylated by CK2 with 200 μM [γ-^{32}P]ATP (0.25 Ci/mmol) as above. Hsp105α was then precipitated with cold 10 % trichloroacetic acid and collected on glass filters, and radioactivity was measured using a liquid scintillation counter. Approx. 0.6 mol/mol of Hsp105α was phosphorylated by CK2 under the experimental conditions.

One-dimensional peptide mapping analysis with lysyl endopeptidase (Lys-C)

Hsp105α (0.5 μg) was phosphorylated by CK2 as described above, and digested with 0.1 μg of Lys-C (Wako) in 20 μl of 100 mM Tris/HCl (pH 9.0) for 3 h at 4 °C. The reaction was terminated by addition of buffer containing 0.1 M sucrose, 3 % SDS, 62.5 mM Tris/HCl, pH 6.9, 5 % (v/v) 2-mercaptoethanol and 0.0025 % (w/v) Bromophenol Blue, and the digests were subjected to SDS/PAGE. After electrophoresis, the peptides were electro-transferred on to PVDF membranes (Bio-Rad), visualized by staining with Coomassie Brilliant Blue R-250 (CBB), and subjected to N-terminal sequencing analysis using a protein sequencer (model 471A; Perkin-Elmer Applied Biosystems).

In vivo phosphorylation and two-dimensional phosphopeptide mapping analysis of Hsp105α

COS-7 cells (6×10^5 cells) in 35 mm dishes were transfected with 1.8 μg of plasmid DNA (pcDNA3 vector, pcDNA105 or pcDNA105S509A) using DMRIE-C (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide and cholesterol; Gibco-BRL) in serum-free Opti-MEM (Gibco-BRL) according to the manufacturer's instructions. At 42 h after transfection, cells were washed with phosphate-free minimal essential medium (Gibco-BRL) and incubated in phosphate-free minimal essential medium supplemented with 10 % fetal calf serum containing 1 mCi/ml [^{32}P]P_i (Amersham Biosciences) for 6 h. Then the cells were washed with PBS, lysed in 200 μl of extraction buffer



(B)

	Size (kDa)	N-terminal amino acid sequence (a.a. residues in mouse Hsp105α)
P1	78	GGSHHHHHHGMA (N-terminal (His-tag))
P2	55	GGSHHHHHHGMA (N-terminal (His-tag))
P3	27	MIMQDKLEKE (622 - 631)
P4	23	VRVNTHGIFTIS (482 - 493)
P5	15	VRVNTHGIFTI (482 - 492)

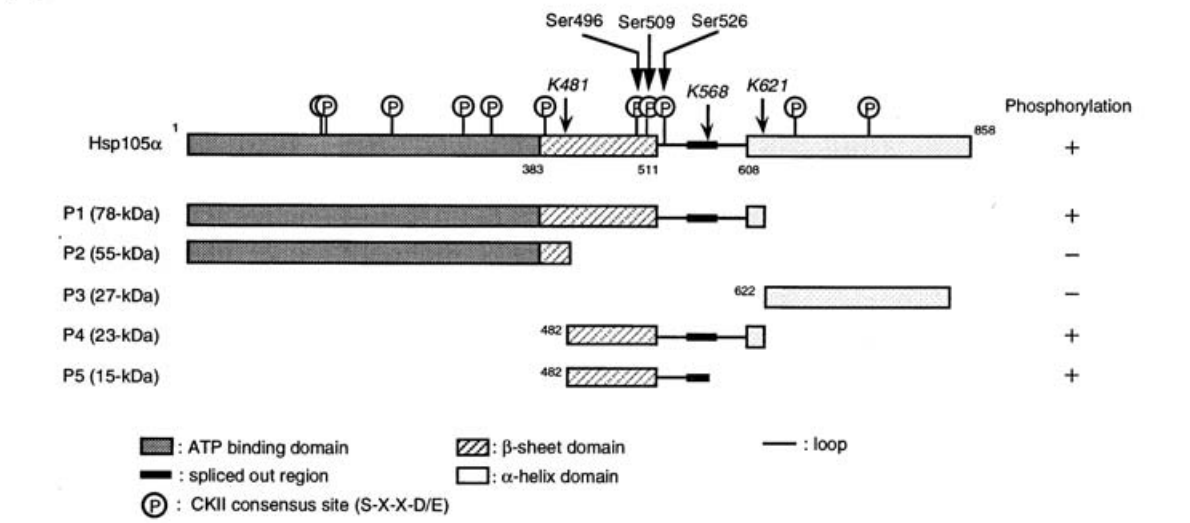


Figure 1 Peptide mapping analysis of Hsp105α phosphorylated by CK2

(**A**) Hsp105α was incubated with CK2 in the presence of [γ-^{32}P]ATP at 37 °C for 3 h, then digested with Lys-C at 4 °C for the indicated times. The digests were separated by SDS/PAGE, and detected by staining with CBB or autoradiography. Numbers on the left represent the molecular masses of marker proteins. Peptides P1–P5 are described in (**B**). (**B**) N-terminal amino acid sequences of P1, P2, P3, P4 and P5 peptides in (**A**) were determined with a protein sequencer. (**C**) Schematic diagram of Lys-C proteolysis of Hsp105α phosphorylated by CK2. Only peptides of which the N-terminal sequence was determined are shown. P in a circle represents the putative CK2 consensus site (Ser/Thr-Xaa-Xaa-Asp/Glu) and Ks represent Lys residues digested by Lys-C. The predicted structures of Hsp105α are also shown: N-terminal ATP binding, β-sheet, loop, C-terminal α-helical domains and a spliced-out region in Hsp105β.

(50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 1 % Nonidet P-40, 20 mM β-glycerophosphate and 2 mM PMSF) for 20 min at 4 °C, and cell extracts were recovered after centrifugation at 12000 ***g*** for 20 min at 4 °C. The cell extracts (200 μg of protein) were mixed with 10 μl of anti-mouse Hsp105 antibody [30], and kept on ice for 1 h. To the mixtures was added 40 μl of 50 % Protein A–Sepharose (Sigma) pre-absorbed with COS-7 cell extracts, followed by rotation for 1 h at 4 °C. After centrifugation at 1000 ***g*** for 3 min, the resulting precipitates were washed five times with extraction buffer containing 0.45 M NaCl, and once with 10 mM Tris/HCl, pH 6.8. To the precipitates was added 40 μl of 2 × SDS-Laemmli sample buffer and the samples were boiled for 5 min, following which the eluted proteins were separated by SDS/PAGE and detected by autoradiography or subjected to two-dimensional phosphopeptide mapping analysis [31].

The *in vivo*-^{32}P-labelled Hsp105α or the *in vitro*-^{32}P-labelled Hsp105α by CK2 was separated by SDS/PAGE and electro-transferred on to nitrocellulose membrane. The ^{32}P-labelled Hsp105α on the membrane was excised and incubated in a solution containing 0.5 % poly(vinylpyrrolidone) and 0.6 % acetic acid for 30 min at 37 °C. The filter pieces were rinsed five

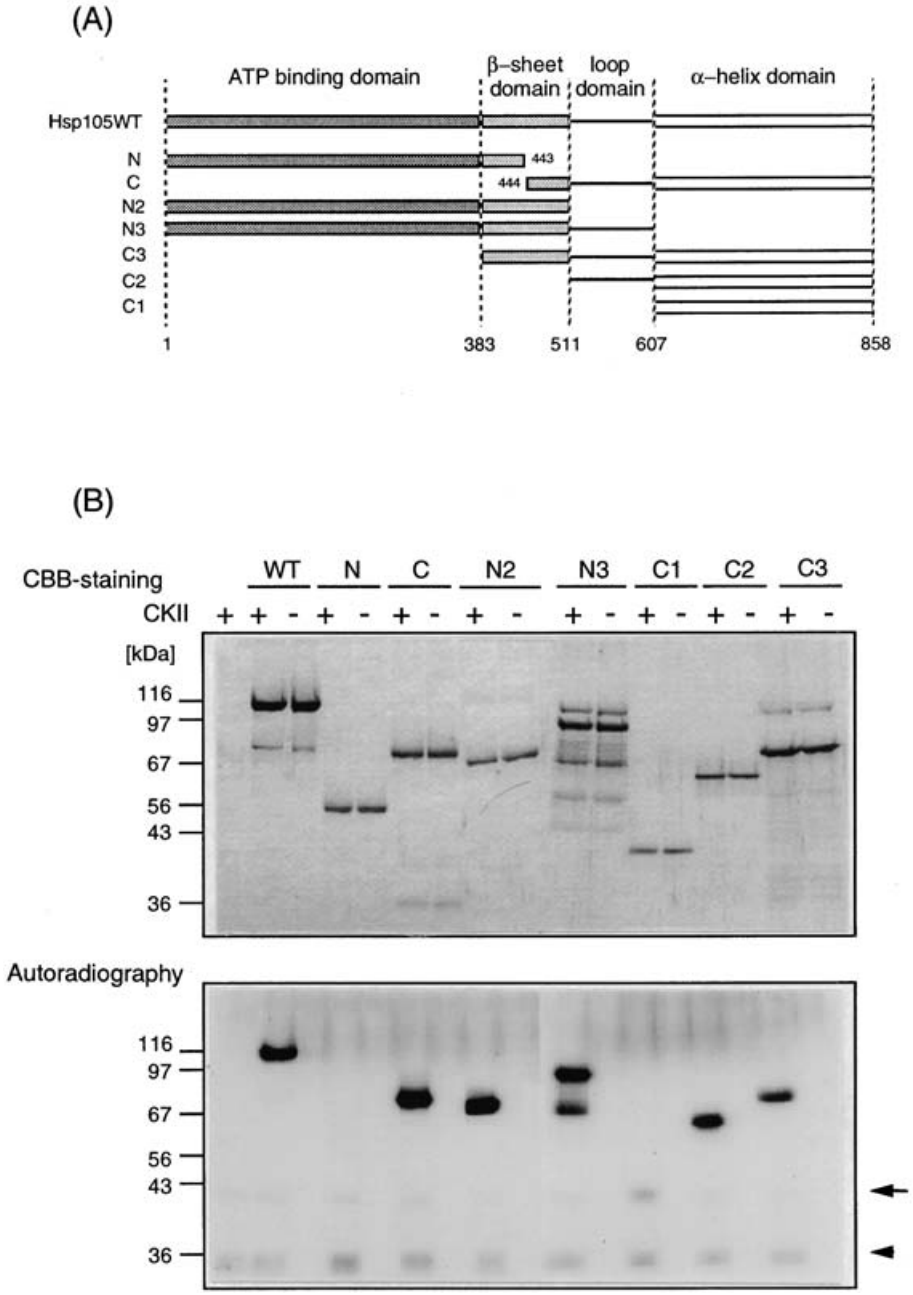


Figure 2 Phosphorylation of deletion mutants of Hsp105α by CK2

(**A**) A schematic diagram of deletion mutants of Hsp105α. (**B**) Hsp105α deletion mutants were incubated with CK2 in the presence of [γ-^{32}P]ATP, separated by SDS/PAGE and detected by staining with CBB or autoradiography. The arrow and arrowhead indicate faint bands of autophosphorylated α and β subunits of CK2, respectively. Numbers on the left represent the molecular masses of marker proteins.

times with water, then incubated in 200 μl of 50 mM ammonium bicarbonate containing 10 μg of Lys-C for 12 h at 37 °C. Peptides released into the supernatant were lyophilized and oxidized in performic acid for 1 h on ice. After an addition of 400 μl of water, the mixture was again lyophilized, dissolved in 200 μl of 50 mM ammonium bicarbonate containing 10 μg of Lys-C and further incubated at 37 °C for 12 h. The reaction mixture was then lyophilized and dissolved in 6 μl of pH 1.9 electrophoresis buffer (formic acid/acetic acid/water, 46:156:1790, by vol.). Phosphopeptides were separated by electrophoresis on cellulose thin-layer plates in the first dimension (1000 V, 30 min), followed by ascending chromatography in the second dimension (isobutyric acid/butan-1-ol/pyridine/acetic acid/water, 1250:38:96:58:558, by vol.). Radioactive phosphopeptides were visualized by autoradiography.

Reactivation of thermally denatured luciferase *in vitro*

Firefly luciferase (164 nM) was incubated with the purified chaperones (2 μM) or BSA in buffer L1 (25 mM Hepes/KOH, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM dithiothreitol and 2 mM ATP) for 5 min at 25 °C, and then denatured for 30 min

at 42 °C. After incubation for 5 min on ice, to 10 μl of the thermally denatured luciferase was added 20 μl of buffer L1 containing 10 mM phosphocreatine, 3.5 units of creatine kinase and 60 % rabbit reticulocyte lysate, after which the mixtures were incubated for the indicated times at 25 °C. Aliquots (2 μl) of the mixture were assayed for luciferase activity (Luciferase assay kit; Promega) using a luminometer (TD-20/20; Turner Designs). Luciferase in the reaction mixtures was detected by immunoblotting using anti-luciferase antibody.

Reactivation of thermally denatured luciferase *in vivo*

The reactivation of thermally denatured luciferase in COS-7 cells was measured according to the method reported by Michels et al. [28]. COS-7 cells (4×10^5 cells) in 60 mm dishes were transfected with 2.5 μg each of pGL2-control and pCMV70 with pcDNA3 vector, pcDNA105WT or pcDNA105S509A. The following day, cells were trypsinized and divided equally into three 35 mm dishes. At 48 h after transfection, cells were pre-treated with 20 μg/ml cycloheximide for 30 min, heat-shocked at 44 °C for 30 min and incubated further at 37 °C for 1 h in the presence of cycloheximide. Cells were then washed with PBS, lysed in 20 μl of Cell Culture Lysis Regent (Promega) and centrifuged at 12000 ***g*** for 5 min at 4 °C. Aliquots (2 μl) of the supernatants were assayed for luciferase activity using a luminometer. Hsp105, Hsp70/Hsc70 and luciferase in the cell lysates (15 μg of proteins) were detected by immunoblotting using anti-human Hsp105 [11], anti-Hsp70 (BRM-22, Sigma) or anti-luciferase (Sigma) antibody.

Western-blot analysis

Proteins were separated by SDS/PAGE (7.5 or 10 % gels) and blotted on to a nitrocellulose membrane. The membrane was incubated with anti-human Hsp105, anti-Hsp70 or anti-luciferase antibody, then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (for Hsp105 and luciferase) or anti-mouse IgG (for Hsc70/Hsp70; both from Santa Cruz Biotechnology). Antibody–antigen complexes were detected using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences).

RESULTS

Identification of phosphorylation site of Hsp105α by CK2 *in vitro*

To identify the phosphorylation site on Hsp105α, we first performed peptide mapping analysis of Hsp105α. Hsp105α was phosphorylated by CK2 in the presence of [γ-^{32}P]ATP *in vitro*, digested with Lys-C, and then the digests were separated by SDS/PAGE and detected by staining with CBB or autoradiography (Figure 1A). Under the conditions used, approx. 0.6 mol/mol of Hsp105α was phosphorylated. Limited proteolysis of Hsp105α resulted in the formation of a number of peptides with a molecular mass in the range 15–78 kDa (Figure 1A). After 7.5 min of digestion, Hsp105α was mainly digested to a peptide of 78 kDa (P1), and smaller peptides were detected in a time-dependent manner. During digestion, radioactive peptides were detected at 78 (P1), 23 (P4) and 15 kDa (P5). To identify peptides P1–P5 in Hsp105α, we determined their N-terminal sequences. As shown in Figure 1(B), peptides P1 and P2 both contained the N-terminal sequence of His-tagged Hsp105α, and the N-terminal amino acid of P3 started from glycine at position 622 of Hsp105α. The N-terminal amino acid sequences of both P4 and P5 started from Val^{482} of Hsp105α.

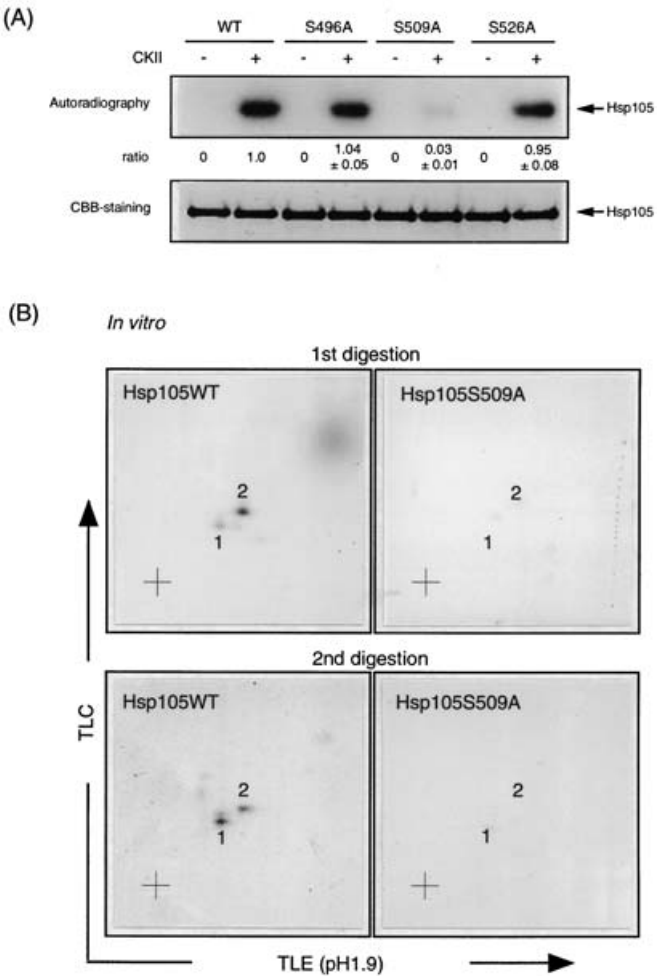


Figure 3 Phosphorylation of substitution mutants of Hsp105α by CK2 *in vitro*

(**A**) Wild-type Hsp105α (Hsp105WT) or mutants with substitution of alanine for serine at positions 496, 509 or 526 (Hsp105S496A, Hsp105S509A and Hsp105S526A, respectively) were incubated with or without CK2 in the presence of [γ-^{32}P]ATP for 3 h at 37 °C, separated by SDS/PAGE, and detected by autoradiography or CBB staining. The density of bands was quantified by densitometry. The relative amounts of ^{32}P incorporated into mutant Hsp105α, normalized by the densities of respective CBB-stained bands, are shown as ratios of those in Hsp105WT. Values represent means ± S.D. from three independent experiments. (**B**) The ^{32}P-labelled Hsp105WT and Hsp105S509A were analysed by two-dimensional phosphopeptide mapping. These Hsp105 was first digested with Lys-C for 12 h (first digestion). The digested peptides were then oxidized in performic acid, and further digested with Lys-C for 12 h (second digestion). The first and second digests were resolved on TLC plates by electrophoresis (TLE) in the first-dimension and chromatography (TLC) in the second dimension.

Figure 1(C) shows the putative phosphorylation sites of CK2 on Hsp105α, and a speculative model of the digestion deduced from the above peptide mapping analysis. Briefly, Hsp105α was digested first to a phosphorylated peptide of 78 kDa, which was the N-terminal peptide of Hsp105α, then to a non-phosphorylated peptide of 55 kDa and a phosphorylated peptide of 23 kDa. The 23 kDa peptide was further digested to a phosphorylated peptide of 15 kDa (Figure 1C). These results suggested that CK2 phosphorylates the central region of Hsp105α.

To further confirm the phosphorylation site, we prepared a series of deletion mutants of Hsp105α, and performed *in vitro* phosphorylation analysis using these mutants. Figure 2(A) shows a schematic diagram of the series of deletion mutants used,

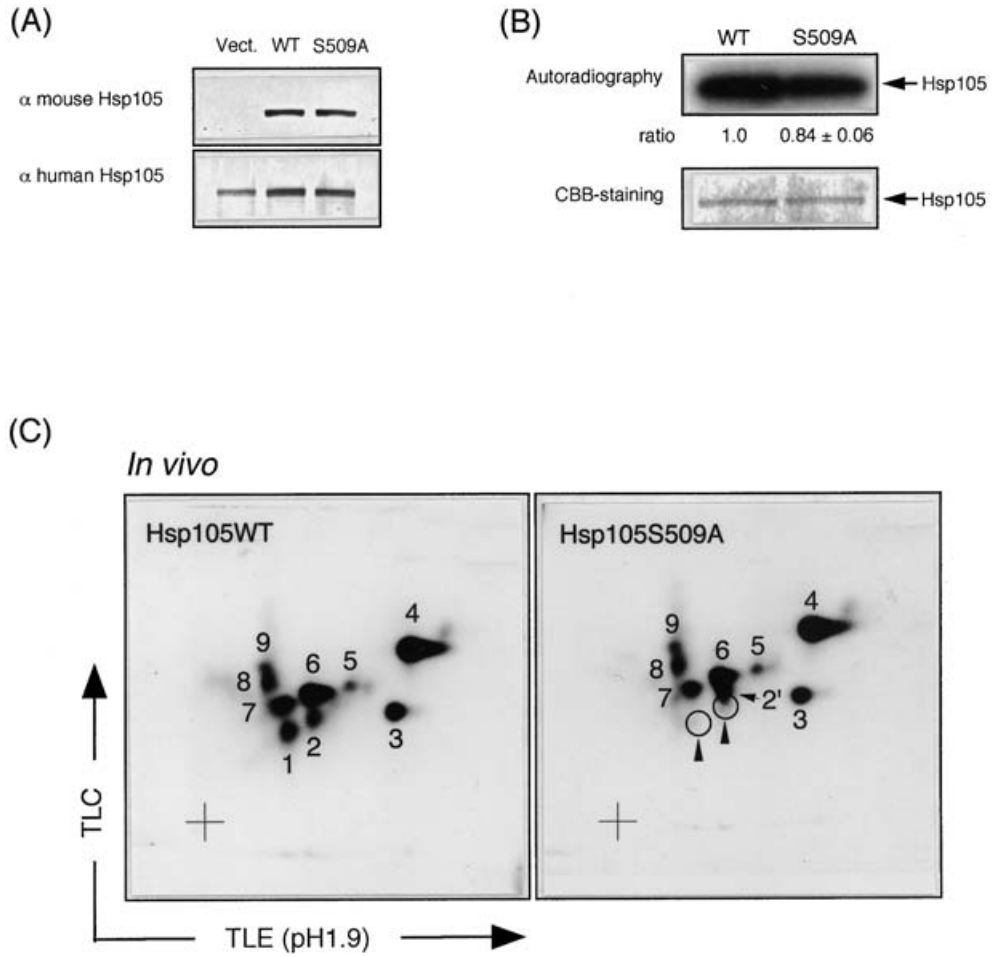


Figure 4 Hsp105α is phosphorylated at Ser^{509} by CK2 *in vivo*

(**A**) COS-7 cells were transfected with pcDNA3 vector, pcDNA105WT or pcDNA105S509A, and 48 h after transfection cells were labelled with [^{32}P]P_i for 4 h. Aliquots (15 μg) of cell extracts were separated by SDS/PAGE, and Hsp105α was detected by immunoblotting using anti-mouse Hsp105 (upper panel) or anti-human Hsp105 (lower panel) antibodies. (**B**) Then Hsp105WT or Hsp105S509A was immunoprecipitated from the cell extracts using anti-mouse Hsp105 antibody, separated by SDS/PAGE, and detected by autoradiography or CBB staining. The density of bands in autoradiographs was quantified and normalized by that of respective CBB-stained bands. Values represent means $\pm$ S.D. from three independent experiments. (**C**) The phosphorylated Hsp105WT and Hsp105S509A in (**B**) were transferred on to nitrocellulose membrane, excised and digested with Lys-C twice and subjected to two-dimensional phosphopeptide mapping as described in the legend to Figure 3. Arrowheads indicate the phosphopeptides corresponding to spots 1 and 2 in Figure 3(B).

and the results are shown in Figure 2(B). CK2 phosphorylated Hsp105N2, Hsp105N3, Hsp105C, Hsp105C2 and Hsp105C3 but not Hsp105N or Hsp105C1, indicating that CK2 phosphorylated Hsp105α in the region between amino acids 444 and 606, consistent with the results shown in Figure 1.

As there are three serine residues as putative CK2 phosphorylation sites in the region between amino acids 444 and 606, we prepared three substitution mutants of Hsp105α in which serine residues at positions 496, 509 and 526 were substituted with alanine residues, designated Hsp105S496A, Hsp105S509A and Hsp105S526A, respectively. As shown in Figure 3(A), when these mutants were phosphorylated by CK2 *in vitro*, Hsp105S496A and Hsp105S526A were phosphorylated similarly to wild-type Hsp105α (Hsp105WT), whereas Hsp105S509A was not phosphorylated at all. Furthermore, when these phosphorylated Hsp105α were digested with Lys-C and analysed by two-dimensional peptide mapping, two phosphopeptide spots (spots 1 and 2) were detected in the CK2-phosphorylated Hsp105WT but not in the CK2-phosphorylated Hsp105S509A (Figure 3B). By the extensive digestion with Lys-C, spot 2 decreased while spot 1 increased, indicating that the spot 2 peptide that contained a phosphorylated Ser^{509} residue was further digested to the spot 1 peptide. These findings strongly suggested that CK2 phosphorylated Hsp105α at Ser^{509} *in vitro*.

In vivo phosphorylation of Hsp105α at Ser^{509}

To determine whether Ser^{509} in Hsp105α is phosphorylated *in vivo*, the mammalian expression plasmid for Hsp105WT or Hsp105S509A was transfected into COS-7 cells, and then the cells were metabolically labelled with [^{32}P]P_i. From the cell extracts, Hsp105WT or Hsp105S509A was immunoprecipitated with anti-mouse Hsp105 antibody [30], separated by SDS/PAGE, and detected by CBB staining or autoradiography. The anti-mouse Hsp105 antibody reacts with mouse Hsp105α but not human Hsp105α [11]. As this antibody also did not recognize monkey Hsp105α in COS-7 cells (Figure 4A), only exogenously expressed mouse Hsp105α was immunoprecipitated in this experiment. As shown in Figure 4(B), Hsp105WT

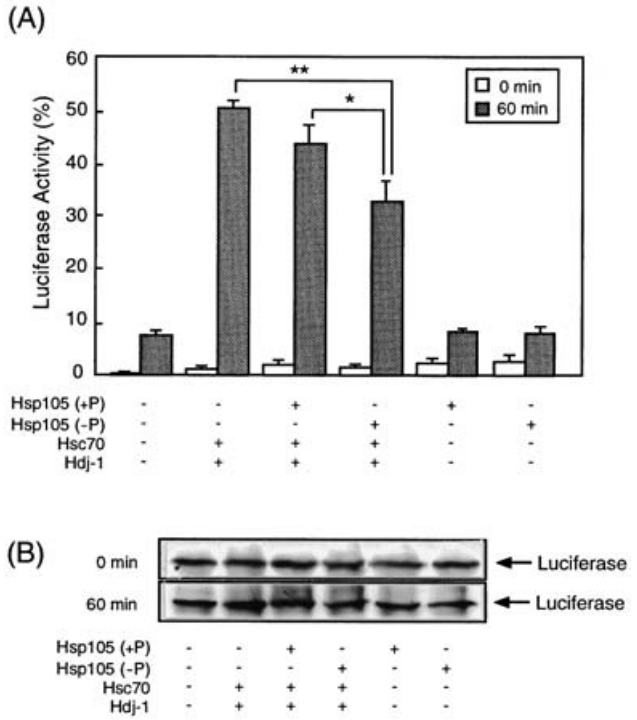


Figure 5 Phosphorylation of Hsp105α suppresses its inhibitory effect on the Hsc70 chaperone activity

(**A**) Hsp105α was incubated with or without CK2 in the presence of 200 μM ATP for 3 h at 37 °C, subjected to gel-filtration chromatography to remove free excess ATP and designated Hsp105(+P) or Hsp105(−P), respectively. Luciferase (164 nM) was incubated in the presence of Hsp105(−P) or Hsp105(+P) alone or in combination with Hsc70/Hdj-1 at 42 °C for 30 min, then incubated with 40 % rabbit reticulocyte lysate at 25 °C for the indicated times. The luciferase activities are expressed as ratios of the original activity. Values represent means ± S.D. from three independent experiments. Statistical significance was determined by Student's *t* test: $^{*}P < 0.05$; $^{**}P < 0.01$. (**B**) Aliquots of the reaction mixtures in (**A**) were separated by SDS/PAGE and luciferase was detected by immunoblotting using anti-luciferase antibody.

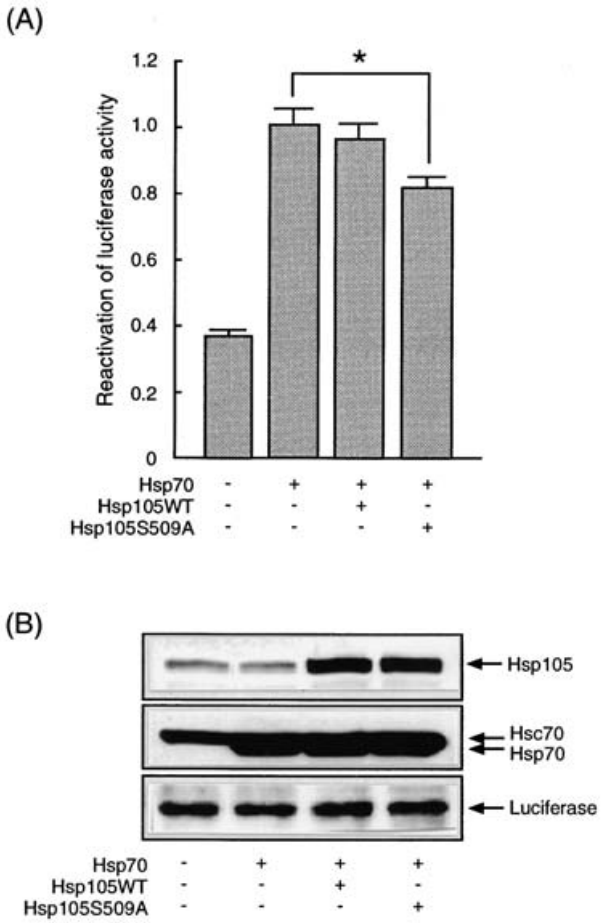


Figure 6 Phosphorylation of Hsp105α at Ser^{509} is important for regulation of the Hsp70 chaperone activity *in vivo*

(**A**) The plasmids pGL2-control and pCMV70 were transiently transfected into COS-7 cells with pcDNA3 vector, pcDNA105WT or pcDNA105S509A. At 48 h after transfection, cycloheximide was added to the medium, and the cells were heat-shocked at 44 °C for 30 min and allowed to recover at 37 °C for 1 h. The reactivation of luciferase represents rates of the Hsp70-dependent increase of luciferase activity after the recovery period. Values represent means ± S.D. from three independent experiments. Statistical significance was determined by Student's *t* test: $^{*}P < 0.05$. (**B**) Aliquots (15 μg) of protein lysates in (**A**) were separated by SDS/PAGE, and proteins were detected by immunoblotting using anti-human Hsp105, anti-Hsp70 or anti-luciferase antibody.

and Hsp105S509A were both phosphorylated, but the level of phosphorylation of Hsp105S509A was approx. 10–20 % lower than that of Hsp105WT. Furthermore, when *in vivo* ^{32}P-labelled Hsp105WT and Hsp105S509A were digested with Lys-C and subjected to two-dimensional phosphopeptide mapping, nine major phosphopeptide spots were detected in Hsp105WT, although the densities of these spots were variable (Figure 4C). Among these spots, spots 1 and 2, found in *in vitro*-CK2-phosphorylated Hsp105WT but not in Hsp105S509A, were clearly absent in the digests of the *in vivo*-phosphorylated Hsp105S509A. However, a new phosphopeptide spot (Figure 4C, spot 2′) was detected at a position close to spot 2 in the digests of Hsp105S509A. Spot 2′ may be a phosphopeptide corresponding to spot 2, in which Ser^{509} was substituted with alanine and phosphorylated by another kinase *in vivo*. Thus Hsp105α was suggested to be phosphorylated at Ser^{509} *in vivo*, although other sites (possibly 7 or less) were also phosphorylated.

Phosphorylation of Hsp105α abolishes its inhibitory activity of the Hsc70/Hsp70 chaperone

As Hsp105α is phosphorylated at Ser^{509} *in vivo* and *in vitro*, we next examined whether phosphorylation of Hsp105α affects the function of this protein. We have shown previously that Hsp105α cannot reactivate thermally denatured luciferase by itself, but suppresses the chaperone activity of Hsc70 *in vitro* [23]. Therefore, we examined the effects of phosphorylation at Ser^{509} by CK2 on the chaperone activity of Hsp105α. Under the experimental conditions used, approx. 0.6 mol/mol of Hsp105α was phosphorylated by CK2. However, phosphorylated Hsp105α alone did not reactivate denatured luciferase similarly to the non-phosphorylated Hsp105α (Figure 5A). Next, we examined the effects of Hsp105α on Hsc70-mediated protein folding. Although non-phosphorylated Hsp105α inhibited Hsc70/Hdj-1-mediated reactivation by rabbit reticulocyte lysate, CK2-phosphorylated Hsp105α was not able to significantly prevent the reactivation of thermally denatured luciferase. CK2 alone did not affect the reactivation activity of Hsc70/Hdj-1 (results not shown). As amounts of luciferase were maintained through the incubation, the reduction of luciferase activity by Hsp105WT was not due to the degradation of luciferase (Figure 5B). Thus phosphorylation of Hsp105α at Ser^{509} seemed to suppress its inhibitory activity for the Hsc70 chaperone.

To confirm these *in vitro* findings, we examined whether phosphorylation of Hsp105α at Ser^{509} is also important for regulation of the Hsp70 chaperone activity *in vivo* (Figure 6A). COS-7 cells transiently overexpressing Hsp70 and luciferase were heat-shocked at 44 °C for 30 min. The luciferase activity in the cells was reduced to approx. 20 % of the original activity. However, after recovery for 1 h the luciferase activity increased to about 45 %. The Hsp70-mediated reactivation of thermally denatured luciferase was not affected by co-expression of Hsp105WT, but was significantly suppressed by co-expression of Hsp105S509A. This reduction of luciferase reactivation was not due to the reduced expression of Hsp70 and luciferase (Figure 6B). As Hsp105WT, but not Hsp105S509A, was thought to be phosphorylated at Ser^{509} in COS-7 cells, the phosphorylation of Hsp105α at Ser^{509} seemed to suppress its inhibition of Hsp70 chaperone activity in mammalian cells. These *in vitro* and *in vivo* findings suggested that the phosphorylation of Hsp105α at Ser^{509} is important for regulation of the Hsc70/Hsp70 chaperone activity.

DISCUSSION

We have shown previously that Hsp105α and Hsp105β exist as phosphorylated and non-phosphorylated forms *in vivo*, and CK2 phosphorylates these proteins *in vitro* [25]. In this study, we showed that Hsp105α was phosphorylated at Ser^{509} *in vitro* and *in vivo*. Hsp105α consists of N-terminal ATP-binding, β-sheet, loop and C-terminal α-helix domains homologous to those of the HSP70 family, and Ser^{509} is located in the β-sheet domain of this protein. Proteins of the HSP70 family transiently interact with substrate proteins through ATP-binding and hydrolysis cycles. The ATP-bound form of Hsp70 has a low affinity for substrates, while the ADP-bound form has high substrate affinity [3,4]. As the β-sheet domain of Hsp70 is important for its substrate-binding activity [4], phosphorylation of Ser^{509} in Hsp105α was thought to affect the function of this protein. In fact, CK2-mediated phosphorylation modulated the inhibitory effect of Hsp105α on Hsc70/Hsp70 chaperone activity.

Hsp105α exists as a complex with Hsp70/Hsc70 and functions as a negative regulator of Hsc70 [23]. Here we showed that phosphorylation of Hsp105α at Ser^{509} by CK2 abolished its inhibition of Hsc70 chaperone activity *in vitro*. As the phosphorylation of Hsp105α by CK2 did not affect the interaction between Hsp105α and Hsc70 (K. Ishihara, N. Yamagishi and T. Hatayama, unpublished work), Hsp105α seems to regulate Hsc70 chaperone activity without dissociating from Hsc70. In addition, the phosphorylation of Hsp105α at Ser^{509} was shown to suppress its inhibitory effect on Hsp70 chaperone activity in mammalian cells, as the Hsp70-mediated reactivation of thermally denatured luciferase was significantly suppressed by co-expression of Hsp105S509A but not Hsp105WT *in vivo*. However, Hsp105S509A was phosphorylated approx. 10–20 % less than wild-type Hsp105α *in vivo* (Figure 4B), and two-dimensional phosphopeptide mapping analysis revealed that Hsp105α seemed to have possibly seven or less phosphorylatable sites *in vivo*, although the extent of phosphorylation was different among them (Figure 4C). Therefore, although phosphorylation of Hsp105α at Ser^{509} seemed to be important for regulation of Hsp70 chaperone activity *in vivo*, Ser^{509} may not have much effect when the other phosphorylation events occur. As phosphorylation is a fundamentally important biological control mechanism, it is important to elucidate the other phosphorylation sites and their function in future studies.

CK2 is a Ser/Thr protein kinase, which is evolutionarily conserved from yeast to human [32], and has been suggested to be involved in cell growth and oncogenesis [33–35], although the precise functions of CK2 are not well understood. More than 100 proteins have been found to be substrates of CK2, and the results of the present study added Hsp105α as a substrate of CK2. The modulation of Hsc70/Hsp70 chaperone activity of Hsp105α by CK2 is a rapid process and may play an important role in cellular events mediated by CK2 in cells.

Although further studies are necessary to understand the molecular mechanism by which phosphorylation of Hsp105α might regulate Hsp105α function, the findings that CK2 phosphorylates Hsp105α at Ser^{509} and modulates the function of Hsp105α may provide clues to elucidating the functions of Hsp105α in mammalian cells. The regulation of Hsp105α function by phosphorylation may play important roles in a variety of cellular events under both stressed and non-stressed conditions.

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